Metabolism of atmospheric hydrogen sulfide in onion

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Chapter 7

Impact of atmospheric H_2S and pedospheric sulfate nutrition on *Allium cepa* - sulfate uptake, expression of sulfate transporters and activity of *O*-acetylserine(thiol)lyase

Abstract. The impact of sulfur nutrition on sulfate uptake was studied in onion (*Allium cepa* L.). A one-week sulfate deprivation resulted in a 1.8-fold increase in sulfate uptake capacity, which was not accompanied by a pronounced induction of sulfate transporter genes. H_2S exposure did neither result in changes in the net sulfate uptake rate nor in the activity of *O*-acetylserine(thiol)lyase (OAS-TL). A nine-day exposure to 0.3 µl l^{-1} H_2S resulted in a 2-fold increase in total sulfur, sulfate and organic sulfur content. Upon cessation of the H_2S exposure the total sulfur content gradually decreased, which could mainly be attributed to growth. The organic sulfur content, however, was decreased to a greater extent than the sulfate content during the first days after the cessation of the H_2S exposure.

Introduction

Sulfur has numerous functions in plants and is present in a variety of compounds. The main proportion is generally present in the protein fraction as cysteine and methionine residues, where it has an important role in the structure, confirmation and function through the formation of disulfide bridges (De Kok *et al*., 2002a). Other plant sulfur pools consist of inorganic sulfate, thiol compounds and sulfolipids, and in certain plant families a substantial amount can be present as secondary sulfur compounds, e.g. glucosinolates in the Brassicaceae and γ-glutamyl peptides and alliins in the Alliaceae. Sulfur-containing compounds are also involved in the regulation of sulfur metabolism and in the protection of plants against a number of stresses, e.g. reactive oxygen species, heavy metals, pathogens. Sulfur deficiency will result in a loss of plant fitness and in a decreased resistance of plants against stress and pests (De Kok *et al*., 2002a,c).

Sulfur is generally taken up as sulfate by the roots, which is mediated by specific sulfate transporter proteins (Hawkesford, 2003; Buchner *et al*., 2004a). Before it can be incorporated, sulfate needs to be reduced to sulfide, a process that mainly takes place in the chloroplast (Droux, 2004; Saito, 2004). Sulfide is subsequently incorporated by the enzyme *O*-acetylserine(thiol)lyase (OAS-TL) into cysteine, which is the direct precursor for most other organic sulfur compounds (Hell *et al*., 2002; Droux, 2004; Saito, 2004). Although root sulfate is the main form of sulfate available for plants in nature, atmospheric sulfur gases, e.g. H_2S, SO_2, can also act as sulfur sources for plant growth (De Kok *et al*., 1997, 2002c). These gases can be useful as a tool to study the regulation of sulfur me-

tabolism and the interaction between shoot and roots. For instance, H_2S is taken up by the stomates and can be directly incorporated as sulfide into cysteine, thereby circumventing the regulational controls of uptake, distribution and reduction of sulfate (De Kok *et al*., 2002a,b).

The impact of H_2S on plant sulfur metabolism differs between species. In *Brassica oleracea* there was a good coordination between the incorporation of H_2S via the shoots and the uptake of sulfate via the roots and its subsequent reduction in the shoot (De Kok *et al*., 2002c; Westerman *et al*., 2000b, 2001b). H_2S could replace sulfate as the source for the formation of organic sulfur, which resulted in a down-regulation/decrease in sulfate uptake and reduction. As a consequence, the total sulfur content was hardly affected upon H_2S exposure in *Brassica oleracea* (Westerman *et al*., 2001a). However, in some species, e.g. *Allium cepa* (onion), H_2S exposure resulted in a substantial increase in the contents of sulfur-containing compounds and sulfate uptake did not seem to be affected (Chapter 4, Durenkamp and De Kok, 2004). Apparently, this species was differently regulated compared to *Brassica oleracea*. In the present study the impact of H_2S exposure and sulfate deprivation on the expression of sulfate transporter genes and sulfate uptake rates was evaluated in onion seedlings.

The observed accumulation of organic sulfur compounds upon H_2S exposure in onion could not be ascribed to an increase in either the protein or the sulfolipid content and likely originated from an increased content of secondary sulfur compounds, viz. γ-glutamyl peptides and alliins (Chapter 3, 4, Durenkamp and De Kok, 2002, 2004; Chapter 6, Durenkamp *et al*., 2005). The increase in the sulfate content could be ascribed to direct oxidation of H_2S and/or degradation of accumulated (secondary) sulfur compounds (Chapter 4, Durenkamp and Kok, 2004). To get more information about the possible degradation of organic sulfur compounds and the subsequent increase in the sulfate content, the decrease in total sulfur, sulfate and organic sulfur content was followed upon cessation of H_2S exposure. The impact of H_2S exposure on the activity of *O*-acetylserine(thiol)lyase was determined, since this enzyme mediates the direct incorporation of H_2S into cysteine and might therefore play a role in the accumulation of sulfur-containing compounds in onion.

Results and discussion

Sulfate uptake and expression of sulfate transporters as affected by sulfur nutrition

The uptake, transport and subcellular distribution of sulfate are mediated by specific sulfate transporter proteins, which can be divided into 5 functionally different groups (Hawkesford, 2003; Buchner *et al*., 2004a,b). Group 1 transporters have a high affinity for sulfate and are generally involved in the initial uptake of sulfate by the roots (Smith *et al*., 1997; Yoshimoto *et al*., 2002). Group 2 transporters are localized in the vascular sys-

tem and are involved in long-distance sulfate transport (Takahashi *et al*., 2000). The role of Group 3 transporters is still unclear, but some evidence exists for an additional role in xylem loading (Kataoka *et al*., 2004a). Vacuolar efflux of sulfate is mediated by Group 4 transporters (Kataoka *et al*., 2004b), whereas the function of Group 5 is not yet known (Buchner *et al*., 2004b). The mechanisms of regulation are still largely unclear and different compounds, e.g. cysteine, glutathione, OAS, sulfate, hormones, are suggested to play a role in the (de)repression of sulfate uptake (Hawkesford, 2000; Maruyama-Nakashita *et al*., 2004). Generally, the expression and activity of the sulfate transporter proteins as well as the actual uptake capacity are strongly affected by the level of sulfur nutrition. Sulfate deprivation generally results in a de-repression of the sulfate transporter genes and a subsequent increase in the sulfate uptake capacity (e.g. Smith *et al*., 1997; Buchner *et al*., 2004a,b; Hopkins *et al*., 2004).

Table 1. The impact of pedospheric and atmospheric sulfur on sulfate uptake in onion (*Allium cepa* L. cv. Nerato). 25-day-old seedlings were either grown in 25 % Hoagland nutrient solution with 0.5 (+S) or 0 (-S) mM sulfate for one week or with 0.5 mM sulfate and exposed to 0 or 0.3 µl l^{-1} H_2S for one week. Sulfate uptake capacity and net sulfate uptake rate were determined over a 24 h and a one-week period, respectively, and expressed on a plant or root basis as µmol g^{-1} fw day^{-1}. Data represent the mean of six measurements with 12 plants in each (± SD). Significant differences between treatments are indicated by asterisks ($P < 0.01$, Student's *t*-test).

	+S	-S	0 µl l^{-1} H_2S	0.3 µl l^{-1} H_2S
Sulfate uptake capacity (24 h)				
Expressed on a plant basis	1.25 ± 0.19	2.27 ± 0.24*		
Expressed on a root basis	5.08 ± 0.88	7.93 ± 1.04*		
Net sulfate uptake rate (7 d)				
Expressed on a plant basis			1.80 ± 0.18	1.69 ± 0.56
Expressed on a root basis			6.11 ± 0.62	5.71 ± 1.87

When onion was deprived of sulfate for one week, it resulted in a 5.5-fold decrease in the sulfate content, a 1.8-fold increase in sulfate uptake capacity on a plant-basis and a slightly decreased shoot to root ratio (Table 1, 2). The same trends could be observed in other species (Smith *et al*., 1997; Westerman *et al*., 2000a; Buchner *et al*., 2004a). Upon re-supply of sulfate for one day, a fast increase in the sulfate content was observed, mainly in the roots (Table 2). A significant part of the sulfate taken up was apparently transported into the root vacuole, where it was temporarily unavailable for transportation to the shoot and subsequent reduction. Three sulfate transporter cDNAs, belonging to Groups 1, 3 and 4, respectively, were isolated from *Allium cepa*. Analysis of the expression patterns of the corresponding mRNAs by Northern hybridization revealed that, in contrast to studies in other species (e.g. Smith *et al*., 1997; Buchner *et al*., 2004a,b; Hopkins *et al*., 2004), the expression of onion sulfate transporters (OnSTs) was hardly influ-

enced by sulfate deprivation (Fig. 1). A slight induction of OnST1.1 could be seen in the roots after a one-week sulfate deprivation (Fig. 1), which might have resulted in the observed increase in the sulfate uptake capacity (Table 1). In *Brassica oleracea* a comparable increase in the sulfate uptake capacity was accompanied by a much stronger induction of Group 1 transporters (Buchner *et al*., 2004a). Apparently, the sulfate uptake system was further regulated on a post-transcriptional level in *Brassica oleracea*. A similar Group 1 transporter was isolated from onion by McCallum *et al*. (2002) and a strong de-repression of gene expression was already apparent after 2 days. Group 3 transporters seem to be the only sulfate transporters that are not responsive to sulfate deprivation (Buchner *et al*., 2004a; Kataoka *et al*., 2004a). OnST3.2 was mainly expressed in the shoot, which might suggest another function than the presumed xylem loading (Fig. 1; Kataoka *et al*., 2004a). OnST4.1 was mainly expressed in the roots and did not respond to sulfate deprivation (Fig. 1), in contrast to observations in other species (Buchner *et al*. 2004a; Kataoka *et al*., 2004b). The impact of sulfate nutrition on the expression of sulfate transporter genes is well characterized in *Brassica* and *Arabidopsis* (Takahashi *et al*., 2000; Buchner *et al*., 2004a) and results are quite similar between these species, since they are strongly related (Buchner *et al*., 2004a). It needs further to be determined whether multiple homologues are also present for the different groups of sulfate transporters in onion as was demonstrated for *Brassica* and *Arabidopsis*.

The impact of atmospheric sulfur nutrition on sulfate uptake is less well characterized than the impact of pedospheric sulfate. SO_2 and H_2S exposure resulted in a decreased uptake of sulfate in duckweed and spinach (Brunold and Erismann, 1974; Schärer *et al*., 1975; Herschbach *et al*., 1995). In *Brassica oleracea* there was a clear interaction between the uptake of H_2S by the shoots and the uptake of sulfate by the roots (Westerman *et al*., 2000a,b; De Kok *et al*., 2002a,c). H_2S could (partly) replace sulfate as the sulfur donor for the synthesis of cysteine and a down-regulation of sulfate uptake was observed. As a result, the total sulfur content was unaffected by H_2S exposure in *B. oleracea*. *Allium* species are assumed to be differently regulated (Chapter 4, Durenkamp and De Kok, 2004). Exposure to H_2S resulted in an accumulation of sulfur-containing compounds in shoots of *Allium cepa*, which depended on the H_2S level and the duration of the exposure (Chapter 6, Durenkamp *et al*., 2005). The total sulfur accumulation upon H_2S exposure was quite similar in sulfate-deprived and sulfate-sufficient plants, which led to the conclusion that sulfate uptake was not down-regulated by H_2S (Chapter 4, Durenkamp and De Kok, 2004). The latter calculation was confirmed when the sulfate uptake rate was determined during a one-week exposure to H_2S (Table 2). In *Brassica oleracea* H_2S exposure mainly resulted in a de-repression of the sulfate uptake system in sulfate-deprived shoots by providing an additional source of sulfur (Buchner *et al*., 2004a). The impact of H_2S on the expression of sulfate transporter genes remains to be investigated in onion.

Table 2. The impact of sulfate deprivation and subsequent re-supply on growth and sulfate content in shoot and roots of onion (*Allium cepa* L. cv. Nerato). 25-day-old seedlings were grown in 25 % Hoagland nutrient solution with 0.5 (+S) or 0 (-S) mM sulfate for one week (T7) and subsequently transferred to a fresh solution with 0.5 mM sulfate for one day (T8). Data represent the mean of three measurements with 12 plants in each (± SD). Significant differences between +S and -S at T7 and T8 are indicated by asterisks ($P < 0.05$ and $P < 0.01$ for growth and sulfate content, respectively, Student's *t*-test).

	Initial	+S (T7)	-S (T7)	+S (T8)	$-S_{resupplied}$ (T8)
Fresh weight					
Shoot (g)	0.24 ± 0.02	0.56 ± 0.02	0.56 ± 0.02	0.66 ± 0.02	0.63 ± 0.06
Root (g)	0.12 ± 0.00	0.21 ± 0.01	0.23 ± 0.02	0.22 ± 0.02	0.26 ± 0.03
S/R ratio	2.0 ± 0.1	2.7 ± 0.1	2.4 ± 0.3	3.0 ± 0.3	2.4 ± 0.2*
Sulfate (μmol g^{-1} fw)					
Shoot	3.6 ± 0.7	4.0 ± 0.3	0.7 ± 0.1*	3.8 ± 0.4	1.3 ± 0.1*
Root	5.0 ± 0.5	4.9 ± 0.7	0.7 ± 0.3*	5.2 ± 0.3	3.4 ± 0.1*

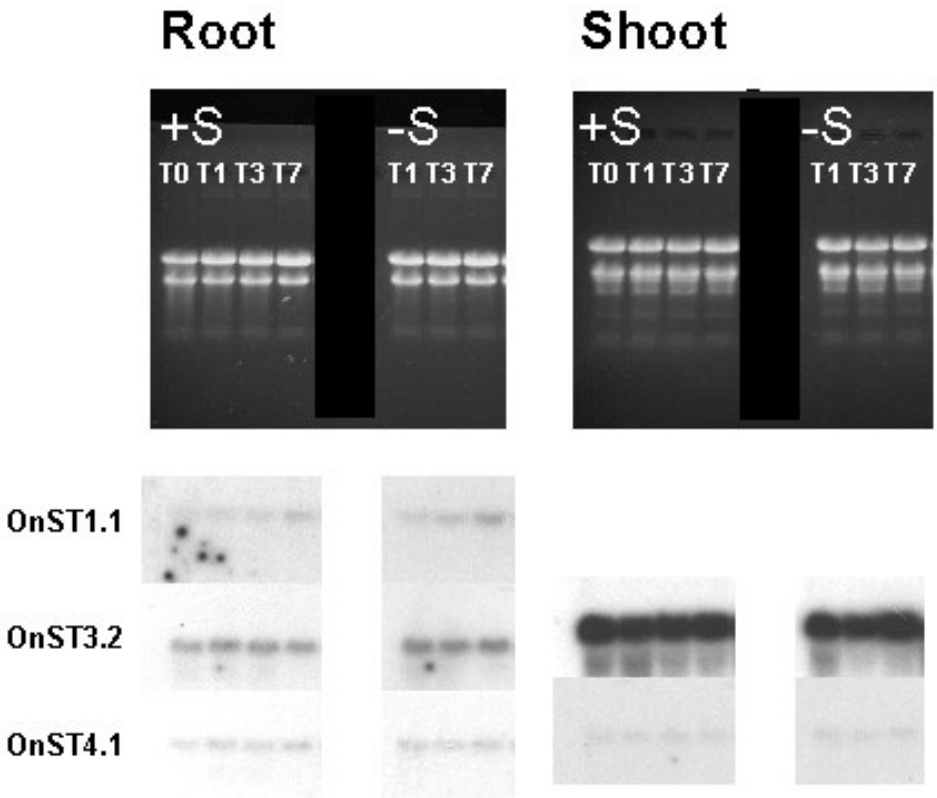

Fig. 1. The impact of sulfate deprivation on the expression of sulfate transporter genes in shoot and roots of onion (*Allium cepa* L. cv. Nerato). 25-day-old seedlings were grown in 25 % Hoagland nutrient solution with 0.5 (+S) or 0 (-S) mM sulfate for one week (T7). 10 μg total RNA was loaded in each slot and separated on an agarose/formaldehyde gel (above). Partial cDNA fragments of Group 1, 3 and 4 were hybridized to the respective onion sulfate transporter (OnST) mRNA (below).

OAS-TL activity is not affected by H_2S exposure

H_2S is directly incorporated into cysteine and the activity of the enzyme *O*-acetylserine(thiol)lyase (OAS-TL), and the availability of its substrate *O*-acetylserine (OAS) are thought to be responsible for the internal (mesophyll) resistance to H_2S and accordingly its deposition rate (Stuiver and De Kok, 2001; De Kok *et al.*, 2002b). Unfortunately, it was not possible to determine the OAS content by HPLC in shoot and roots of onion, which was due to interference by an unknown compound, specific for Allium species (M. Wirtz, personal communication). In order to determine if the accumulation of sulfur compounds upon H_2S exposure was due to an enhanced activity of OAS-TL, the latter was measured in onion seedlings, which were exposed to 0.3 µl l^{-1} H_2S for one week. The activity of OAS-TL in shoot and roots was not affected by H_2S exposure (Table 3), which indicated that the H_2S deposition rate depended on other factors, e.g. the availability of OAS and/or the activity of the OAS-synthesizing enzyme serine acetyltransferase (SAT). OAS-TL and SAT are complexed into cysteine synthase (Hell, 2003; Droux, 2004; Wirtz *et al*., 2004). SAT is thought to be active only in the complexed form, whereas OAS-TL is active as free enzyme. In general, OAS-TL is available in excess to SAT (about 300-fold in chloroplasts; Droux *et al*., 1998). Therefore, changes in activity of OAS-TL are not likely to have a major impact on rate of synthesis of cysteine, although they could have an effect on the formation of the cysteine synthase complex. The activity of OAS-TL was also not affected upon H_2S exposure in *Brassica oleracea* (Westerman *et al*., 2001b), but in this species an accumulation of sulfur compounds could neither be observed (Westerman *et al*., 2001a). The increased OAS-TL activity on a protein basis in the roots when compared to the shoot (Table 3) was caused by an approx. 2.5-fold lower protein content in the roots (data not shown).

Table 3. Impact of H_2S exposure on the activity of *O*-acetylserine(thiol)lyase (OAS-TL) in shoot and roots of onion (*Allium cepa* L. cv. Nerato). Four-week-old seedlings were grown in 25 % Hoagland nutrient solution and exposed to 0 and 0.3 µl l^{-1} H_2S for nine days. Data are expressed on a fresh weight (µmol g^{-1} fw h^{-1}) and a protein basis (µmol mg^{-1} protein h^{-1}) and represent the mean of three measurements with 12 plants in each (± SD). No significant differences between treatments were found ($P < 0.01$, Student's *t*-test).

	0 µl l^{-1} H_2S	0.3 µl l^{-1} H_2S
Expressed on fresh weight		
OAS-TL activity, shoot	333 ± 5	330 ± 18
OAS-TL activity, root	414 ± 32	394 ± 33
Expressed on protein content		
OAS-TL activity, shoot	61 ± 2	60 ± 5
OAS-TL activity, root	199 ± 25	190 ± 8

H_2S exposure resulted in an accumulation of sulfate in sulfate-deprived plants, which could be caused by the degradation of accumulated secondary sulfur compounds and/or direct oxidation of H_2S (Chapter 4, Durenkamp and De Kok, 2004). In *Brassica oleracea*, H_2S partly replaced sulfate as sulfur source for growth and down-regulation of both sulfate uptake and reduction via APS reductase prevented an accumulation of sulfate (Westerman *et al*., 2000a, 2001b; De Kok *et al*., 2002c). From the present data, it is evident that H_2S did not affect the sulfate uptake by the roots (Table 1; Chapter 4, Durenkamp and De Kok, 2004). However, H_2S exposure might result in a decreased sulfate reduction and plants might transfer from pedospheric sulfate to atmospheric H_2S as sulfur source for organic sulfur compounds in the shoot. This might be one of the causes for the observed increase in the sulfate content upon H_2S exposure in sulfate-sufficient plants, as well as for the relatively faster decrease in the organic sulfur content after cessation of H_2S exposure (Fig. 2). The uptake of sulfate and its reduction by APS reductase are thought to be the main regulation points in the assimilation of sulfur (Vauclare *et al*., 2002). It remains to be investigated to what extent H_2S exposure affects the enzymes involved in sulfate reduction (mainly APS reductase).

Cessation of H_2S exposure rapidly decreases the organic sulfur content

The accumulation and decrease of sulfate and organic sulfur were studied upon exposure to H_2S at different seedling ages and upon cessation of exposure, respectively. Onion seedlings were exposed to 0 or 0.3 μl l^{-1} H_2S for nine days (pretreatment). Subsequently, the non-exposed plants were exposed to 0 (0/0) or 0.3 (0/0.3) μl l^{-1} H_2S for six days and the H_2S-exposed plants were exposed to 0 (0.3/0, cessation) or 0.3 (0.3/0.3) μl l^{-1} H_2S for six days (Fig. 2). Onion was not very susceptible to the toxic effects of H_2S (Chapter 3, 5, Durenkamp and De Kok, 2002, 2005; Chapter 6, Durenkamp *et al*., 2005), since growth was not affected by any of the treatments (data not shown). Sulfur contents in the roots were not affected by both H_2S exposure and subsequent cessation of exposure (data not shown; Chapter 3, 4, Durenkamp and De Kok, 2002, 2004). H_2S exposure for nine days resulted in a 2.0, 2.2, and 1.9-fold increase in total sulfur, sulfate and organic sulfur content of the shoot, respectively (Fig. 2). Accumulation of sulfur-containing compounds upon H_2S exposure was observed in all species and cultivars of *Allium* (Chapter 3, Durenkamp and De Kok, 2003). A decrease in the total sulfur content was observed upon cessation of H_2S exposure, which was most apparent in the first three days (Fig. 2). This decrease could largely be explained by growth, since the slopes of the cessation treatment (0.3/0) and the non-exposed treatment (0/0) were comparable when expressed on a plant basis (Fig. 2). A decrease in accumulated sulfate upon cessation of SO_2 exposure could also be explained by dilution in content due to growth (Maas *et al*., 1987). From day 9 to 12, total sulfur, sulfate and organic sulfur content were decreased by 25, 20 and 28 %, respectively, whereas from day 12 to 15, this decrease was 8, 10, and 6 %, respectively (Fig.

2). Therefore, changes in the level of H_2S exposure in the first place led to variations in the organic sulfur content and afterwards in the sulfate content. The amount of organic sulfur on a plant basis hardly increased after cessation of H_2S exposure from day 9 to 12 (Fig. 2), therefore, the decrease in its content could only partly be explained by growth. The additional decrease remains to be investigated, but could have resulted from degradation of accumulated organic (secondary) sulfur compounds, volatilization of organic sulfur compounds or a reduced reduction of sulfate due to H_2S exposure.

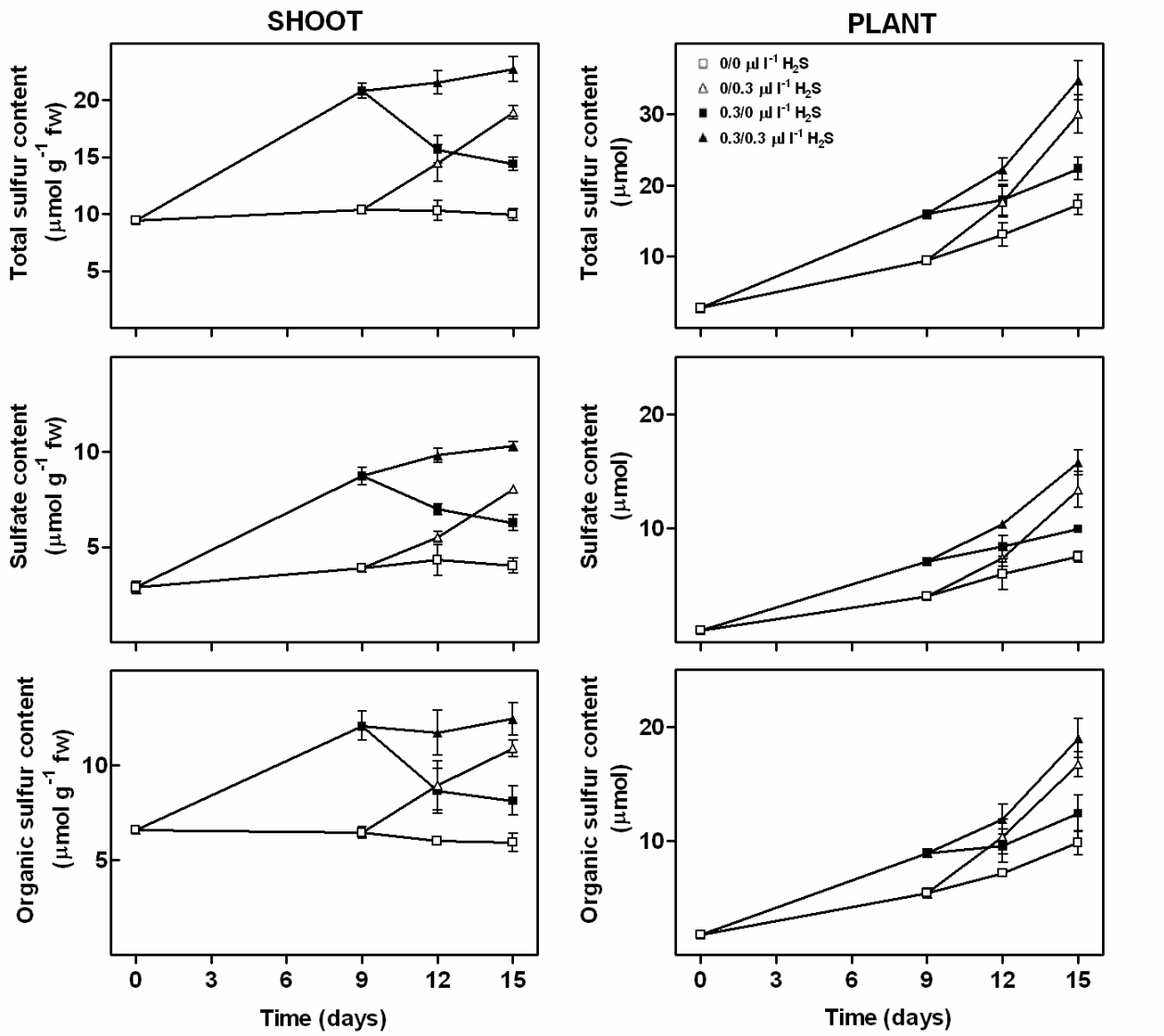

Fig. 2. Impact of H_2S exposure and subsequent cessation of exposure on total sulfur, sulfate and organic sulfur content in onion (*Allium cepa* L. cv. Nerato). 24-day-old seedlings were grown in 25 % Hoagland nutrient solution and exposed to 0 (open symbols) and 0.3 (closed symbols) µl l^{-1} H_2S for nine days and both treatments were subsequently exposed to 0 (squares) and 0.3 (triangles) µl l^{-1} H_2S for another six days. Data are expressed in µmol g^{-1} fw shoot (left) and µmol $plant^{-1}$ (right) and represent the mean of three measurements with 12 plants in each (± SD).

Durenkamp and De Kok (2005, Chapter 5) observed that the accumulation of sulfur-containing compounds was less pronounced in the second week of exposure to H_2S, which is confirmed by the present data (Fig. 2). This could be explained by a relative decrease in the uptake of H_2S in older plants (due to changes in morphology), by the possible release of volatile (secondary) sulfur compounds (Kanda and Tsuruta, 1995) and/or by a decrease in the sulfate uptake rate by the roots (Westerman *et al*., 2000a). The present data do not support the first option. When control plants were exposed to H_2S after day 9 (Fig. 2), a substantial increase in total sulfur content in the shoot was observed, which was comparable to the accumulation upon H_2S exposure from day 0 to 9 (Fig. 2). This indicated that the reduced accumulation during long-term exposure could not be ascribed to differences in developmental stage and morphology of the plants, but likely had to originate from volatilization of secondary sulfur compounds and/or a decreased uptake of sulfate. Although the present results did not indicate an impact on sulfate uptake rate upon exposure to H_2S for one week (Table 1; Chapter 4, Durenkamp and De Kok, 2004), a decrease upon long-term exposure could not be excluded.

The accumulation of sulfate upon H_2S exposure in sulfate-deprived plants was an indication of direct oxidation of H_2S and/or degradation of accumulated organic (secondary) sulfur compounds to sulfate (Chapter 4, Durenkamp and De Kok, 2004). The present data did not provide conclusive evidence for either cause and more research will be needed to determine the nature of the H_2S-induced sulfate accumulation.